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STRAINS OF CUCUMBER MOSAIC VIRUS PATHOGENIC ON CRUCIFERS [1]

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INTRODUCTION

In recent years the ornamental crucifer, dames violet (*Hesperis matronalis* L.) has been reported as being susceptible to a number of strains of turnip virus 1 (*3, 4, 6, 8, 9, 11*) [3] but nonsusceptible to other strains of turnip virus 1 (*4, 10*) and to strains of cauliflower virus 1 (*7, 12*) under artificial inoculations. Although McWhorter [4] reported a mosaic disease of dames violet from Oregon in 1936, no study of viruses naturally affecting this host has been made.

In 1942, the senior author made inoculations from plants of dames violet growing at Madison, Wis., and affected with a mosaic disease, the symptoms of which resembled those caused by the cabbage A strain of turnip virus 1 (*12*). However, instead of the local necrotic lesions on tobacco (*Nicotiana tabacum* L. var. Havana 38) characteristic of turnip virus 1 (*2*), a systemic distorting mottle not unlike that caused by cucumber virus 1 was produced. In 1946, a slightly different strain of this latter virus was isolated from naturally infected plants of dames violet growing in another section of Wisconsin. This host has been found since to be widely infected with this virus throughout much of Wisconsin.

The purpose of this paper is to report experiments by which these viruses were identified as strains of cucumber virus 1 and to describe their symptoms on selected hosts.

MATERIALS AND METHODS

Studies were carried out in greenhouses which were fumigated frequently to control insects. Stock cultures of the viruses were carried in plants of *Nicotiana glutinosa* L. kept in insectproof cages. Frequent inoculations were made to healthy plants to provide inoculum for study. Greenhouse air temperatures were kept at 20° to 24° C. except as otherwise mentioned. Host plants were grown in 4-inch clay pots in greenhouse compost. In making the host range study, four or five plants were inoculated in each test and an equal number of uninoculated plants were kept as controls. The presence or absence

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[2] The writers wish to express appreciation to J. B. Kendrick, Jr., for making some of the greenhouse inoculations and to Eugene Herrling for making the photographs used in this manuscript.

[3] Italic numbers in parentheses refer to Literature Cited p. 12.

[4] MCWHORTER, F. P. MOTTLING OR BREAKING IN DAME'S ROCKET IN OREGON. U. S. Bur. Plant Indus., Plant Dis. Rptr. 20: 199. 1936. [Processed.]

of the virus in each test was confirmed by mechanical transfer from the host in question to tobacco which gave a characteristic symptom. Mechanical inoculations were made by sprinkling the plants with powdered carborundum and then by applying sap extract of virus-stock plants by means of a glass spatula. Properties of the viruses were determined according to methods previously described (*12*).

The virus strain originally obtained from dames violet in 1942 is referred to herein, for convenience, as strain X1 and the similar isolate obtained in 1946 is referred to as strain X2.

EXPERIMENTAL RESULTS

SYMPTOMS AND HOST RANGE

Symptons on dames violet in nature consist of a conspicuous chlorotic vein clearing accompanied by a pronounced yellow-green or bright yellow, coarse mottling (fig. 1). Curvature of midribs and

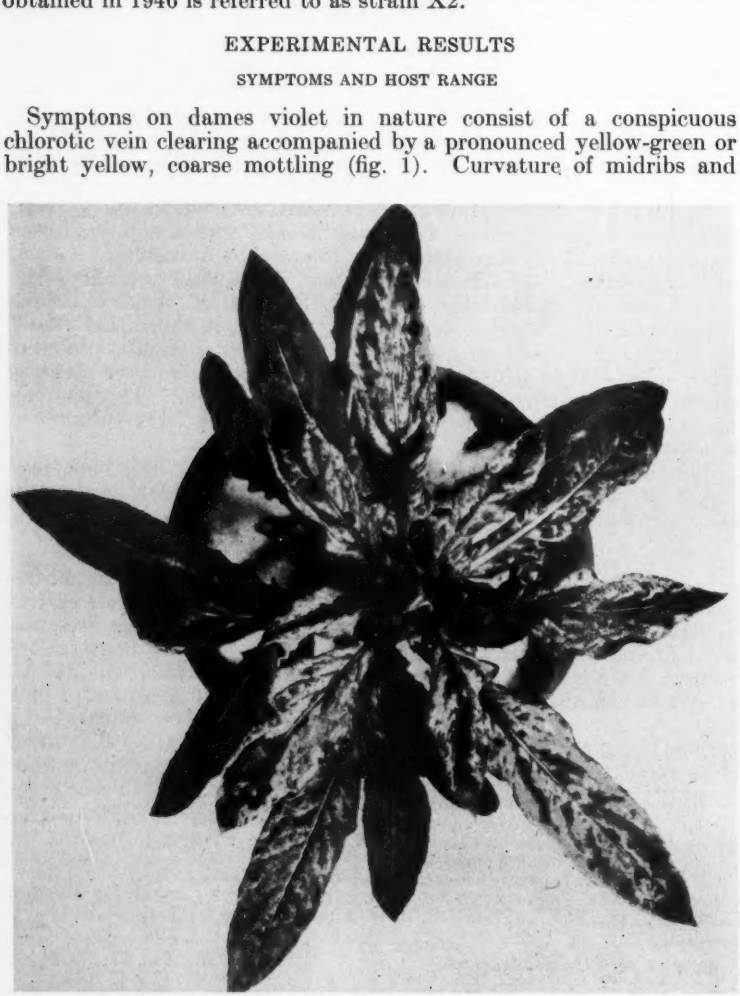

FIGURE 1.—Systemic symptoms produced on dames violet by the X1 strain of cucumber virus 1.

puckering of laminae are common and stunting is severe (fig. 2). Purplish necrosis often outlines the periphery of chlorotic lesions. Sectional or complete breaking of flowers accompanies infection. Symptoms produced on greenhouse plants are similar to those observed in nature but usually are less pronounced. The effects of strains X1 and X2 do not differ greatly from those produced by cabbage virus A (*12*); it is difficult to differentiate between them except by clinical tests (fig. 2).

On tobacco, symptoms of strains X1 and X2 were identical and appeared in 5 to 8 days as a chlorotic vein clearing followed by yellow-green mottling. Plants were mildly to moderately stunted and showed little of the leaf malformation so characteristic of many strains of cucumber mosaic virus. The mottling appeared first as circular chlorotic lesions which, with age, graded into irregular chlorotic patches between the veins and irregular segments of normal dark-green tissue along the veins (fig. 3). With age, infected plants tended to recover somewhat from symptoms.

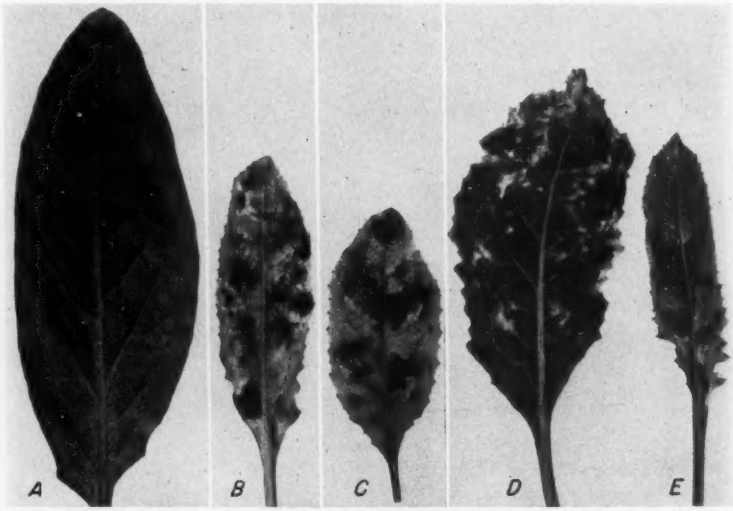

FIGURE 2.—Systemic symptoms produced on dames violet by the X1 strain of cucumber virus 1 and by the cabbage A strain of turnip virus 1: *A*, Leaf from healthy plant; *B*, *C*, leaves from plant infected with cucumber virus X1; *D*, *E*, leaves from plant infected with cabbage virus A. Note the similarity of symptoms produced by the two unrelated viruses.

In table 1 are given the host range and a brief description of symptoms of strains X1 and X2 as determined in greenhouse experiments. It can be seen that both viruses were widely pathogenic among cruciferous plants but that conspicuous symptoms were not produced on members of *Brassica oleracea* L. In general, the wild crucifers, including dames violet, shepherds-purse, pennycress, upland cress, and the wild mustards showed prominent symptoms (figs. 4, 5). The two viruses were separable on these hosts by minor differences in symptom type and intensity.

FIGURE 3.—Systemic symptoms produced on tobacco by the X1 strain of cucumber virus 1.

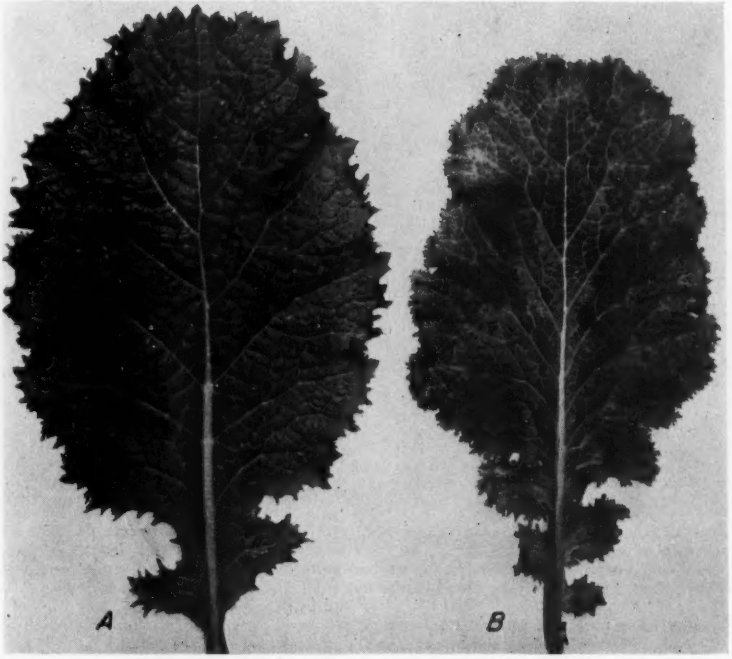

FIGURE 4.—Systemic symptoms produced on Indian mustard by the X2 strain of cucumber virus 1 (B). Note stunting and coarse mottling when compared with leaf from healthy plant (A).

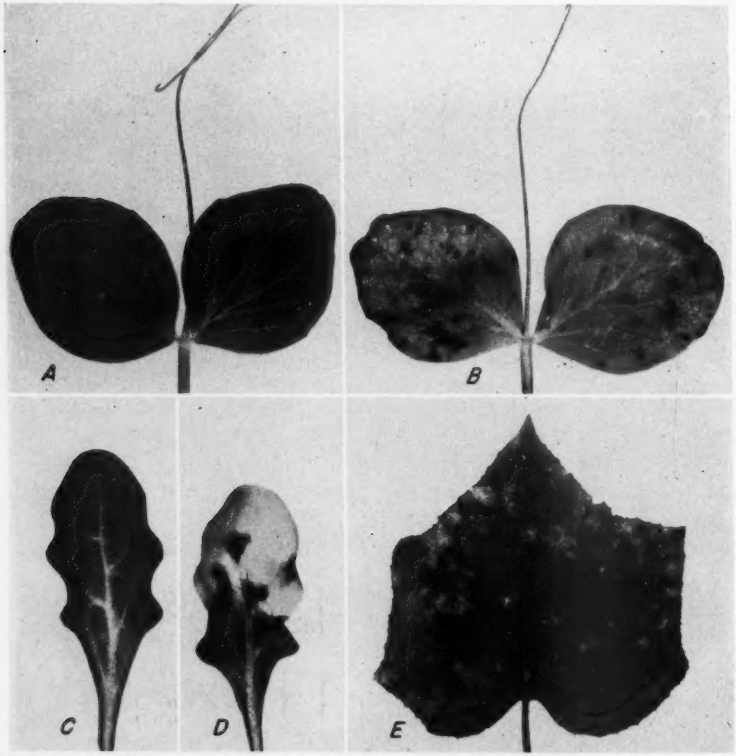

FIGURE 5.—Symptoms produced on pea, pennycress, and cucumber by the X1 strain of cucumber virus 1: *A*, *B*, uninoculated and inoculated leaves of pea, respectively; *C*, *D*, uninoculated and inoculated leaves of pennycress, respectively; *E*, leaf from systemically infected cucumber.

TABLE 1.—*Local and systemic symptoms produced by the X1 and X2 strains of cucumber virus 1 on selected hosts in the greenhouse*

Host family and species	Symptoms produced by	
	X1	X2
Amaranthaceae:		
Amaranthus caudatus L. (love-lies-bleeding).	Local: Marked reddish-brown necrotic lesions; leaf abscission. Systemic: Chlorotic mottling becoming necrotic; stunting; distortion.	Local: As for X1. Systemic: None; virus not recovered.
Compositae:		
Aster amellus L. (aster)	Local: Slight chlorosis and necrosis Systemic: Slight chlorosis; stunting.	As for X1.
Calendula officinalis L. (calendula, var. Orange King).	Local: None Systemic: None; virus recovered.	Local: None. Systemic: Faint mottling.
Cichorium intybus L. (chicory)	Local: None Systemic: None.	As for X1.
Dahlia pinnata Cav. (dahlia)	Local: Circular chlorotic lesions Systemic: Chlorosis; stunting; diffuse mottling.	Local: As for X1. Systemic: Mottling less pronounced but leaf distortion more pronounced than for X1.

TABLE 1.—*Local and systemic symptoms produced by the X1 and X2 strains of cucumber virus 1 on selected hosts in the greenhouse*—Continued

Host family and species	Symptoms produced by	
	X1	X2
Compositae—Continued		
Helianthus annuus L. (sunflower).	Local: Scattered necrotic lesions Systemic: Mild mottling; stunting.	As for X1.
Zinnia elegans Jacq. (zinnia, var. Liliput).	Local: None Systemic: Chlorotic mottling; stunting; distortion.	Not tested.
Convolvulaceae:		
Ipomoea purpurea (L.) Roth. (morning-glory).	Local: Irregular necrotic lesions Systemic: Conspicuous coarse mottling; severe stunting; distortion.	Local: As for X1. Systemic: None.
Cruciferae:		
Barbarea vulgaris R. Br. (upland cress).	Local: None Systemic: Mild, diffuse mottling; marked stunting.	Local: None. Systemic: Conspicuous, blotchy, chlorotic vein banding; marked stunting.
Brassica juncea (L.) Coss. (Indian mustard).	Local: None Systemic: Slight chlorosis; mottling.	Local: None. Systemic: Conspicuous blotchy mottling; stunting; more pronounced than for X1.
B. kaber (DC.) Wheeler (charlock).	Local: None Systemic: Vein clearing and mild mottling.	Local: None. Systemic: Mild, whitish mottling; slight leaf distortion.
B. napus L. (rape, var. Dwarf Essex).	Local: None Systemic: Slight chlorosis.	As for X1.
B. nigra (L.) Koch (black mustard).	Local: Slight chlorosis Systemic: Slight chlorosis; chlorotic vein banding.	Local: As for X1. Systemic: Chlorotic vein banding; blotchy mottling; more pronounced than X1.
B. oleracea var. *acephala* DC. (Siberian kale).	Local: None Systemic: None; virus recovered.	As for X1.
B. oleracea var. *botrytis* L. (broccoli, var. Italian Green Sprouting).	Local: Slight chlorosis Systemic: None; virus recovered.	Do.
B. oleracea var. *botrytis* L. (cauliflower, var. Snowball).	Local: Slight chlorosis Systemic: None; virus recovered.	Do.
B. oleracea var. *capitata* L. (Cabbage, var. Jersey Queen).	Local: None Systemic: None; virus recovered.	Do.
B. oleracea var. *gemmifera* Zenker (brussels sprouts, var. Long Island Mammoth).	Local: Slight chlorosis Systemic: None; virus recovered.	Do.
B. oleracea var. *gongylodes* L. (kohlrabi, var. Purple Vienna).	Local: None Systemic: None; virus recovered.	Do.
B. pekinensis (Lour.) Rupr. (Chinese cabbage, var. Chihli).	Local: Occasional chlorotic lesions Systemic: None.	Local: None. Systemic: Vein banding; blotchy mottling.
Capsella bursa-pastoris (L.) Medic. (shepherds-purse).	Local: None Systemic: Slight chlorosis; marked stunting.	Local: None. Systemic: Mild mottling; necrosis; stunting.
Cheiranthus allionii Bailey (Siberian wallflower).	Local: None Systemic: Mild stunting	As for X1.
Erysimum cheiranthoides L. (worm-seed mustard).	Local: None Systemic: None; virus recovered.	Do.
Hesperis matronalis L. (dames violet).	Local: None Systemic: Conspicuous yellow-green or yellow mottling; stunting; distortion; symptoms appear only after 6 to 8 weeks' incubation.	Do.
Lobularia maritima (L.) Desv. (sweet alyssum).	Local: Chlorosis Systemic: General chlorosis; stunting.	Do.
Matthiola incana var. *annua* (L.) Voss (stock, var. Dwarf Flowering Ten Weeks).	Local: None Systemic: Slight stunting.	Do.
Sisymbrium officinale (L.) Scop. (hedgemustard).	Local: Scattered necrotic lesions Systemic: Mild mottling.	As for X1 but more pronounced.
Thlaspi arvense L. (penny-cress).	Local: Conspicuous necrotic lesions and necrotic streaking of veins. Systemic: Moderate stunting.	As for X1 but appear later and develop more slowly.
Cucurbitaceae:		
Citrullus vulgaris Schrad. (watermelon, var. Early Kansas).	Local: Necrotic lesions Systemic: None; virus not recovered.	Not tested.
Cucumis melo L. (muskmelon, var. Golden Delicious).	Local: General chlorosis becoming necrotic. Systemic: Mild mottling; stunting.	Local: As for X1. Systemic: Slight chlorosis only.

TABLE 1.—*Local and systemic symptoms produced by the X1 and X2 strains of cucumber virus 1 on selected hosts in the greenhouse*—Continued

Host family and species	Symptoms produced by	
	X1	X2
Cucurbitaceae—Continued		
C. sativus L. (cucumber, vars. Maine, Ohio 31).	Local: General chlorosis. Systemic: Mild diffuse mottling, tending to become masked with age; stunting. On variety Ohio 31 only slight chlorosis, very faint mottling and slight stunting, much less than for Maine.	Local: As for X1. Systemic: Only mild stunting.
Cucurbita maxima Duchesne (Squash, var. Blue Hubbard).	Local: Chlorosis followed by necrosis. Systemic: None; virus not recovered.	Local: Slight chlorosis. Systemic: None; virus not recovered.
C. pepo L. (sugar pumpkin)	Local: Blotchy chlorotic lesions becoming necrotic. Systemic: None; virus not recovered.	As for X1.
Gramineae:		
Zea mays var. *rugosa* Bonaf. (sweet corn, var. Golden Bantam).	Local: Streak chlorosis becoming necrotic. Systemic: Necrosis of meristematic tissue; rapidly lethal.	Local: None. Systemic; None; virus recovered.
Leguminosae:		
Phaseolus vulgaris L. (bean, var. Stringless Green Refugee).	Local: None Systemic: None	As for X1.
Pisum sativum L. (pea, vars. Alaska, Prince of Wales).	Local: Conspicuous necrotic lesions; leaf abscission. Systemic: None	Local: General chlorosis; leaf abscission; develop 5 to 7 days slower than for X1. Systemic: None.
Polemoniaceae:		
Phlox drummondii Hook. (phlox).	Local: None Systemic: Slight chlorosis; stunting	As for X1.
Resedaceae:		
Reseda odorata L. (mignonette).	Local: None Systemic: Very mild mottling	As for X1 but slightly more pronounced.
Scrophulariaceae:		
Antirrhinum majus L. (snapdragon).	Local: None Systemic: Slight chlorosis; stunting	Local: None. Systemic: Chlorosis; mild mottling; stunting.
Solanaceae:		
Capsicum frutescens var. *cerasiforme* (Mill.) Bailey (pepper, var. California Wonder)	Local: None Systemic: Conspicuous chlorotic mottling; stunting; distortion.	Local: None. Systemic: None.
Datura metel L.	Local: Necrotic lesions. Systemic: Slight mottling; necrosis	As for X1 but slightly more severe.
Lycopersicon esculentum Mill. (tomato, var. Bonny Best)	Local: Scattered pin-point necrotic lesions. Systemic: Mild mottling with no distortion.	As for X1.
Nicandra physalodes (L.) Pers. (apple-of-Peru)	Local: Numerous necrotic lesions with rapid leaf abscission. Systemic: Severe necrosis; extreme stunting.	Do.
Nicotiana glutinosa L.	Local: Scattered chlorotic lesions. Systemic: Vein clearing followed by yellow-green mottling; severe stunting and distortion.	Local: None. Systemic: Bleached chlorosis; mild stunting; much less severe than X1.
N. rustica L.	Local: Broad, zonate necrotic lesions. Systemic: Mild mottling; slight distortion.	Local: Numerous pin-point necrotic lesions. Systemic: None.
N. tabacum L. (tobacco, var. Connecticut Havana 38).	Local: None Systemic: Vein clearing followed by green mottling; very slight distortion.	As for X1 but shows more recovery from symptoms.
Petunia hybrida Vilm. (petunia).	Local: Chlorotic rings becoming necrotic. Systemic: Severe mottling; severe stunting and distortion.	As for X1.
Physalis pubescens L. (husk tomato).	Local: Chlorotic rings. Systemic: Chlorotic rings producing mottling effect; stunting; distortion.	Not tested.
Solanum integrifolium Poir. (Chinese eggplant).	Local: Broad chlorotic lesions. Systemic: Diffuse chlorotic mottling; stunting.	Do.
S. triflorum Nutt.	Local: Severe development of small necrotic lesions; rapid abscission. Systemic: All plants rapidly killed by spreading necrosis.	As for X1 but slightly less severe.

It is interesting that neither virus developed systemically in squash, pumpkin, or watermelon although each produced local symptoms. However, systemic invasion of watermelon by strains of cucumber virus 1 is not common. Symptoms produced on cucumber (var. Maine) by strain X1 were very mild and consisted only of mild mottling and stunting (fig. 5, *E*). Strain X2 produced no symptoms other than mild stunting but could be recovered from inoculated plants. The Ohio 31 cucumber, which has been released as resistant to cucumber mosaic, was very tolerant to strains X1 and X2, showing almost no symptoms.

It can be seen from a study of table 1 that strains X1 and X2 are very similar, yet distinct in several respects. Thus, on pepper, love-lies-bleeding, and morning-glory, strain X1 produced conspicuous systemic symptoms while strain X2 produced no systemic symptoms and did not develop systemically in love-lies-bleeding (fig. 6). On

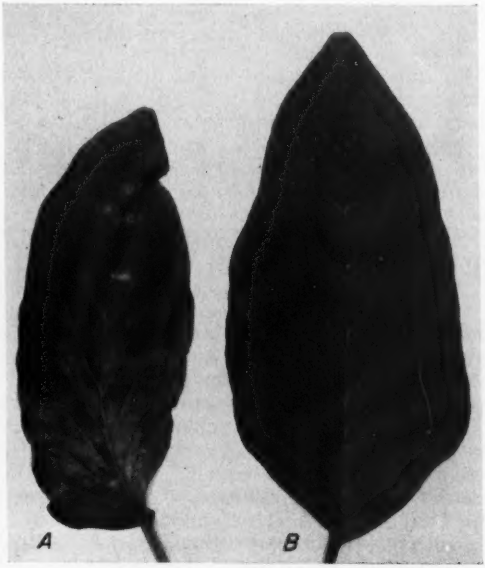

FIGURE 6.—Systemic symptoms produced by the X1 and X2 strains of cucumber virus 1 on *Amaranthus caudatus*. *A*, leaf from plant infected with strain X1; *B*, leaf from plant inoculated with strain X2 in which no systemic invasion occurred.

sweet corn strain X1 produced severe necrosis, resulting in rapid death of the plants; strain X2 developed systemically but produced no symptoms. Ultimate symptoms of the two viruses on pennycress were identical but those of strain X2 appeared 5 to 7 days later than those of strain X1. On *Barbarea vulgaris*, *Brassica juncea*, *B. nigra*, and other wild mustards, the two viruses were indistinguishable except for slight differences in symptom type and intensity.

PROPERTIES

The results in table 2 show that the X1 and X2 strains each had properties similar to those of cucumber virus 1 but that they were less tolerant to aging in vitro than most strains of that virus. The points at which inactivation occurred were identical for strains X1 and X2 and are listed as follows: Tolerance to dilution, 1 to 100,000; thermal inactivation, 70° C. for 10 minutes; aging in vitro, 4 to 5 days at 20° C.

TABLE 2.—*Physical properties of strains X1 and X2 of cucumber virus 1 as determined on tobacco*

[10 plants inoculated with each virus for each treatment]

Type and degree of treatment	Plants infected with strain—		Type and degree of treatment	Plants infected with strain—	
	X1 [1]	X2 [1]		X1 [1]	X2 [1]
Dilution:	*Number*	*Number*	Heating (°C. for 10 min.)—Continued	*Number*	*Number*
0	10	10	60	6	7
1-10	10	10	65	2	2
1-100	10	10	70	0	0
1-1,000	8	10	Aging in vitro (days at 20°):		
1-10,000	2	3	0	10	10
1-100,000	0	0	1	10	10
Heating (°C. for 10 min.):			2	9	10
None	10	10	3	1	4
45	10	10	4	1	0
50	10	10	5	0	0
55	10	10			

[1] Values given for X1 are averages of 3 trials; those for X2 represent only 1 trial.

CLASSIFICATION OF STRAIN X1

Price (*5*) has shown that zinnia plants infected with any mottle-producing strain of cucumber virus 1 are immune to his strain No. 6, which produced primary necrotic lesions. Since strain X1 produced a systemic mottle in zinnia, the No. 6 strain [5] was tested with strain X1 as follows. Ten zinnia plants were inoculated with strain X1, and after all plants were thoroughly mottled, 4 systemically infected leaves on each plant were reinoculated with strain 6. At the same time an equal number of healthy plants of the same age similarly were inoculated with strain 6. One necrotic lesion developed on the 40 leaves previously infected with strain X1 and reinoculated with strain 6, while 364 lesions developed on 40 leaves of the healthy plants inoculated with strain 6. In a second trial, involving the same number of plants, no lesions developed on the plants previously infected with X1, while 213 lesions developed on healthy plants inoculated with strain 6. In still a third trial, the lesion counts were 0 and 180, respectively. These results would seem to demonstrate a strain relationship between X1 and strain 6.

No cross immunity test was made involving strain X2 but because of its close similarity to X1 throughout these studies it is also considered to be a strain of cucumber virus 1.

[5] Kindly supplied by Dr. W. C. Price.

DISCUSSION

In the many cases of selective pathogenicity cited for the cucumber mosaic virus group and recently reviewed by Whipple and Walker (*13*), little evidence has been reported for pathogenicity within the Cruciferae. Ainsworth (*1*) mentioned finding a cucumber virus affecting water cress. Instances of cucumber mosaic viruses infecting various crucifers under artificial inoculations have been reported, but in no case has any specific virus been found to be widely infectious within the family. The viruses described in this paper were infectious to every crucifer tested, including 19 species in 10 different genera. It is important that no economically important species of the genus *Brassica* produced symptoms of any prominence. These as well as several other crucifers tested could be classed as symptomless carriers. In view of the very mild symptoms produced on cultivated crucifers these viruses are not considered of economic importance on these crops.

Strains X1 and X2 have a wide host range, one or the other affecting every species tested except bean. Several of their reactions are notable. In general their effects on cucurbits were very mild, only local symptoms being produced on squash, pumpkin, and muskmelon. Strain X2 produced only very mild symptoms on cucumber. The mild symptoms on cucurbits serve to separate them from many strains of cucumber virus 1. The production of only local symptoms on pea and the failure to infect bean separate strains X1 and X2 from strains 14 and 17 of cucumber virus 1 also described from Wisconsin by Whipple and Walker (*13*). Strains X1 and X2 also produced mild symptoms on tobacco and tomato with little or no distortion on either.

The physical properties of strains X1 and X2 agree in general with those described for cucumber virus 1. In view of this close agreement and the fact that strain X1 effectively immunized zinnia plants against infection with Price's strain 6 of cucumber virus 1, it appears logical to consider strain X1 and X2 as belonging to the cucumber virus 1 group.

SUMMARY

A mosaic disease of the ornamental crucifer, dames violet (*Hesperis matronalis* L.), is described herein. Two causal viruses which are very closely related are identified as strains of cucumber virus 1. Both viruses were found to be widely pathogenic on members of the Cruciferae, but no prominent symptoms were produced on cultivated species of this family other than dames violet. Descriptions of symptoms and host range of the two viruses are given.

The physical properties of the two viruses agreed closely with those of cucumber virus 1. Points at which inactivation occurred were: Tolerance to dilution, 1 to 100,000; thermal inactivation, 70° C. for 10 minutes; aging in vitro, 4 to 5 days at 20° C.

One of the two viruses was shown to effectively immunize zinnia plants against Price's No. 6 strain of cucumber virus 1.

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PRODUCTION OF DERRIS ELLIPTICA IN RELATION TO TYPE OF CUTTING AND AGE AT HARVEST [1]

By DAVID G. WHITE, formerly *associate plant pathologist*, CALEB PAGÁN, *junior chemist*, and MERRIAM A. JONES, formerly *associate chemist*, *Puerto Rico (Mayaguez) Federal Experiment Station, United States Department of Agriculture* [2]

INTRODUCTION

The roots of *Derris elliptica* (Wall.) Benth. are used as a source of insecticidal compounds, primarily rotenone. Generally, plants are propagated from leafless cuttings of the vines and are harvested after 2 years in the field. (*6*, *8*).[3] Cooper (*2*) found that treatment of leafless cuttings with root-promoting substances, such as 3-indolebutyric acid, caused profuse rooting. However, most of the additional roots died before shoots were well developed and there was no lasting advantage from the treatment. He also found that leafy cuttings could be made from small stem material usually discarded. Rooting of the leafy cuttings was augmented by treatment with root-promoting substances and the additional roots continued to develop, thereby resulting in a more fibrous root system in the early stages than that from cuttings not treated. As a result of this work an experiment was started in late July 1943 [4] to compare the production of roots, rotenone, and related compounds in plants propagated from leafless and leafy cuttings and harvested at several ages.

MATERIALS AND METHODS

Mature vines of the Sarawak Creeping variety were used as a source of propagating material. Small leafy cuttings 3 to 4 mm. in diameter and about 15 cm. in length bearing three leaf blades each (fig. 1) were prepared July 29 and 30, 1943. Half of these were treated by dipping the basal ends for 5 seconds in a solution of 5 mg. of 3–indolebutyric acid per milliliter of 50-percent alcohol (*2*). The treated cuttings and controls were planted July 30 and 31 in the greenhouse in shaded sand beds covered with sash to maintain high humidity. Leafless cuttings were prepared in three groups according to diameter: (*1*) Small stem, 3 to 4 mm. in diameter and about 30 cm. in length; (*2*) medium stem, 5 to 6 mm. in diameter and about 30 cm. in length; and (*3*) large stem,

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[2] Appreciation is expressed to O. A. Pope, principal biometrician, Office of Foreign Agricultural Relations, U. S. Department of Agriculture, Washington, D. C., for his advice in making the statistical analyses.

[3] Italic numbers in parentheses refer to Literature Cited, p. 23.

[4] The experiment was initiated by William C. Cooper, Rufus H. Moore, and Merriam A. Jones.

8 to 12 mm. in diameter and about 23 cm. in length (fig. 1). These cuttings were planted in field nursery beds of soil between August 3 and 5.

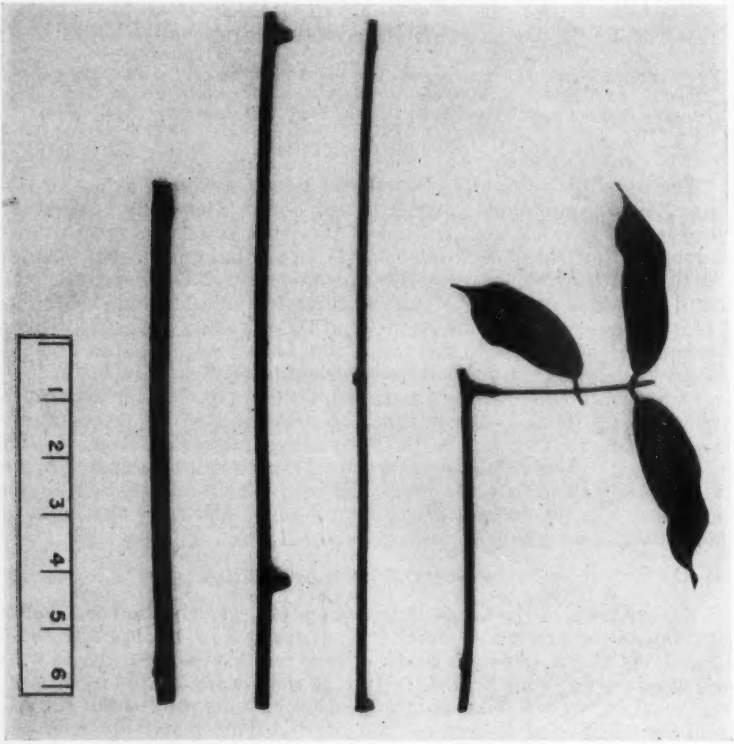

FIGURE 1.—Different types of stem cuttings of the Sarawak Creeping variety of *Derris elliptica*. From left to right: Large, medium, and small leafless cuttings and a small leafy cutting.

The small leafy cuttings, both treated and controls, appeared well rooted 17 days after planting, when 4 replicates of 32 plants each of the treated and control leafy cuttings were removed from the sand bed for root analyses. At the same time the remaining cuttings were transplanted to field nursery beds, where partial shade was supplied for 3 days. The treated leafy cuttings averaged 50 roots each and the controls averaged 5. The first harvest of the 3 sizes of leafless cuttings in the field nursery was made 24 days after planting, using 4 replicates of 32 plants each. A second harvest was made from the field nursery beds 2 months after planting and a third harvest was made a month later. Leafless cuttings of medium diameter were not included in the third harvest because only sufficient material remained for planting in the field.

Three months after the cuttings were planted those which had not been harvested were transplanted to a field of Toa clay loam at distances of 2 feet apart in rows of 3 feet apart without trellising. Plants from each type of cutting were transplanted to plots of 25, 5 plants long and 5 plants wide. The plots were located in a randomized block design whereby 5 plots of each type of cutting were replicated within 4 blocks. Five months later, or 8 months after the original nursery plantings, the roots of the 9 centrally located plants in 1 plot of each type of cutting in each of the 4 blocks were harvested to a depth of 16 inches. The 16 plants comprising a border row which surrounded the 9 sample plants of each plot were not dug. At intervals of 6 months thereafter 4 other plots of each type of cutting were harvested up to 32 months after the nursery plantings.

After each harvest the roots were washed, separated into diameter groups as described later, then cut into pieces 1 inch in length, and allowed to dry under shelter. Air-dry weights were recorded after about 1 month, when no further fluctuations in weight occurred. Samples were next ground in a Wiley mill through a 0.5 mm. screen followed by colorimetric analysis of each sample for rotenone plus rotenoids (3).

The data from each treatment for each harvest were analyzed for variance, and significant differences were established by the *t* test. Analyses of variance for the combined data of the last four harvests were made similarly.

RESULTS

The average percentages of rotenone plus rotenoids in roots developed from each type of cutting and at each harvest are presented in table 1. Significant and highly significant differences existed among the different types of cuttings within each harvest except in harvests made 26 and 32 months after planting. Most often roots of the control leafy and large size leafless cuttings contained significantly higher rotenone plus rotenoids than roots from the other three types of cut-

TABLE 1.—*Average content of rotenone plus rotenoids in roots of Derris elliptica grown from different types of cuttings and harvested at different ages*

Age at harvest	Type of cutting					Significant differences within each harvest		
	A Control leafy	B Treated leafy	C Small leafless	D Medium leafless	E Large leafless	*t* at 5 percent	*t* at 1 percent	Rank [1]
	Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	
24 days	0.8	0.8	2.0	1.1	0.7	1.1	1.5	C>A, B, E.
2 months	3.4	3.0	1.7	2.0	3.5	.4	.6	A, B, E,>>C, D.
3 months	7.2	8.5	3.6	(2)	7.6	1.5	2.1	A, B, E,>>C.
8 months	8.7	9.3	6.0	6.2	6.9	2.5	3.5	A, B>C, D.
14 months	14.7	15.3	13.3	15.0	17.1	3.5	4.9	E>C.
20 months	15.6	14.8	11.4	11.7	16.1	2.7	3.8	A, E,>>C, D, B>C, D,
26 months	15.8	17.5	14.8	15.6	17.4	3.8	5.3	None.
32 months	14.7	14.3	14.8	12.8	13.9	2.6	3.6	None.

[1] The sign ">" indicates significantly greater than (odds 19:1) and the signs ">>" indicate greater than by high significance (odds 99:1).
[2] Insufficient number of plants.

tings. The average of all roots from each harvest is shown graphically in figure 2.

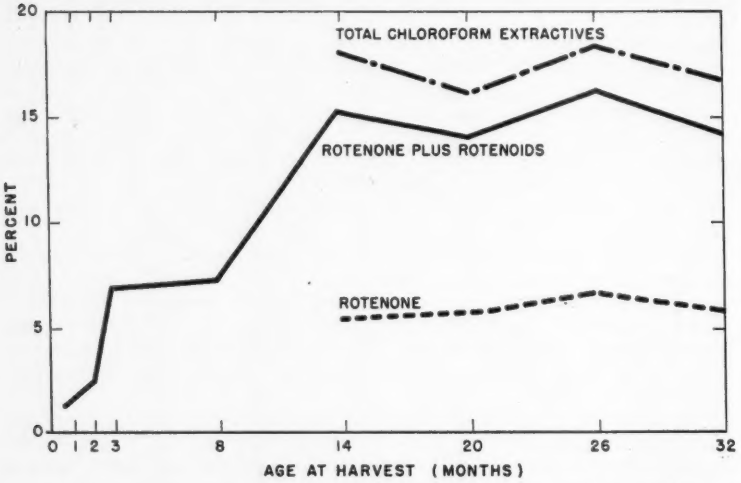

FIGURE 2.—The average percentage of rotenone, rotenone plus rotenoids, and total chloroform extractives in relation to age at harvest.

The rotenone in derris roots is roughly proportional to the red-color value which is indicative of the content of rotenone plus rotenoids. However, this relationship must be determined for each set of samples. Prior to the harvest 14 months after planting, the root samples were of insufficient quantity for separate determinations of rotenone. Beginning with that harvest, however, rotenone determinations of the roots of each plot were made by the official method (*1, p. 64*). The averages of these data are presented in table 2. Significant and highly significant differences existed among types of cuttings within each harvest except in the harvest made 26 months after planting.

TABLE 2.—*Average rotenone content of roots of Derris elliptica grown from different types of cuttings and harvested at several ages*

Age at harvest (months)	Type of cutting					Significant differences within each harvest		
	A Control leafy	B Treated leafy	C Small leafless	D Medium leafless	E Large leafless	*t* at 5 percent	*t* at 1 percent	Rank [1]
	Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	
14	5.10	5.55	4.38	5.08	5.93	1.20	1.68	E>C.
20	6.18	5.50	4.58	4.55	6.28	1.07	1.48	A, E>>C, D.
26	6.60	7.18	5.80	6.15	7.18	1.54	2.15	None.
32	6.17	5.40	5.90	5.05	5.43	1.03	1.44	A,>D.

[1] The sign ">" indicates significantly greater than (odds 19:1) and the signs ">>" indicate greater than by high significance (odds 99:1).

The average rotenone content of all roots from each of these harvests is shown graphically in figure 2.

The total chloroform extractives in derris roots are also roughly proportional to the content of rotenone and of rotenone plus rotenoids. In this experiment 10-ml. aliquots of the chloroform extracts used in the rotenone determinations were removed. The aliquots were evaporated on a steam bath followed by drying in an oven at 105° C. to constant weight. The average total chloroform extractives on a percentage basis are presented in table 3 for the last four harvests. Significant and highly significant differences existed among types of cuttings within each harvest except in the harvest made 32 months after planting. The average total chloroform extractives of all roots in the last four harvests are shown graphically in figure 2.

TABLE 3.—*Average total chloroform extractives in roots of Derris elliptica from different types of cuttings and harvested at several ages*

Age at harvest (months)	Type of cutting					Significant differences within each harvest		
	A Control leafy	B Treated leafy	C Small leafless	D Medium leafless	E Large leafless	*t* at 5 percent	*t* at 1 percent	Rank [1]
	Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	
14	17.7	18.7	16.1	17.9	20.5	4.2	5.8	E>C.
20	17.7	16.6	13.2	13.5	18.4	3.6	5.0	E>>C, D; A>C,D.
26	18.4	20.4	16.0	16.4	19.8	4.3	5.9	B>C.
32	18.0	16.6	17.1	15.6	16.5	2.7	4.3	None.

[1] The sign ">" indicates significantly greater than (odds 19:1) and the signs ">>" indicate greater than by high significance (odds 99:1).

The oven-dry weights of roots in each plot were determined and the data were then calculated to yield in pounds per acre, as shown in table 4. During the first 8 months after planting more roots were

TABLE 4.—*Average yield per acre of dry roots of Derris elliptica grown from different types of cuttings and harvested at several ages*

Age at harvest	Type of cutting					Significant differences within each harvest		
	A Control leafy	B Treated leafy	C Small leafless	D Medium leafless	E Large leafless	*t* at 5 percent	*t* at 1 percent	Rank [1]
	Pounds	*Pounds*	*Pounds*	*Pounds*	*Pounds*	*Pounds*	*Pounds*	
24 days	0.186	0.425	0.003	0.006	0.060	0.036	0.050	B>>A, C, D, E; A>>C, D, E, E>>C, D.
2 months	1.74	2.30	.03	.07	.23	.28	.38	B>>A, C, D, E; A>>C, D, E.
3 months	4.24	3.74	.78	[2]	.84	1.19	1.66	A, B,>>C, E.
8 months	16.2	19.4	3.8	5.2	12.2	7.9	11.0	A, B,>>C, D, E>C.
14 months	150	172	151	185	197	98	136	None.
20 months	685	814	725	725	615	201	280	Do.
26 months	732	739	644	725	713	254	352	Do.
32 months	1,007	1,097	1,134	939	1,039	318	443	Do.

[1] The sign ">" indicates significantly greater than (odds 19:1) and the signs ">>" indicate greater than by high significance (odds 99:1).

[2] Insufficient number of plants.

usually produced from the leafy cuttings than from the leafless. In the first 2 months after planting the treated leafy cuttings yielded more roots than any other type of cutting. However, no differences among types of cuttings occured in the harvest made at 14 months after planting and thereafter. The average yields of roots at each harvest are shown graphically in figure 3.

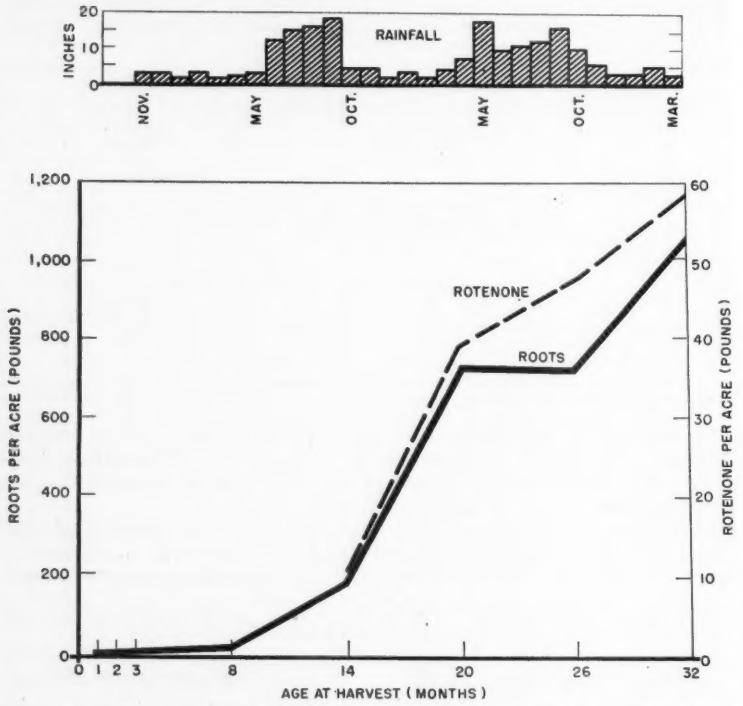

FIGURE 3.—The average yields of air-dry roots and rotenone in pounds per acre in relation to age at harvest. The upper graph shows the monthly rainfall during the 29 months that the plants were in the field. The months at the beginning and end of this period are shown, as are those at the beginning and end of each of the two 6-month rainy seasons.

The yield of rotenone per acre in the last four harvests was calculated from the weight of dry roots multiplied by the percent of rotenone and these data were analyzed to establish the significance of differences, as shown in table 5. Little difference among types of cuttings within harvests occurred. Although small leafless cuttings produced the least amount of rotenone in the harvest at 26 months, they produced the greatest amount 6 months later. The average yields of rotenone per acre at each harvest are shown graphically in figure 3.

Analyses of the combined data for the last four harvests were made to determine the significance of differences caused by interaction of the

TABLE 5.—*Average yield of rotenone per acre of Derris elliptica roots grown from different types of cuttings and harvested at several ages*

Age at harvest (months)	Type of cutting					Significant differences within each harvest		
	A Control leafy	B Treated leafy	C Small leafless	D Medium leafless	E Large leafless	*t* at 5 percent	*t* at 1 percent	Rank [1]
	Pounds	*Pounds*	*Pounds*	*Pounds*	*Pounds*	*Pounds*	*Pounds*	
14	8.3	10.0	6.8	9.5	12.2	6.5	9.1	None.
20	42.1	44.2	33.5	33.2	38.9	13.4	18.6	Do.
26	48.2	53.7	38.2	44.3	50.8	13.6	18.8	B>C.
32	62.1	56.0	66.9	47.1	56.5	17.4	24.2	C>D.

[1] The sign ">" indicates significantly greater than (odds 19:1).

age at harvest and the type of cutting on yields of roots, rotenone, and percentage of rotenone. The sums required for analysis are presented in tables 6 and 7. There was no gain in weight of roots per acre between 20 and 26 months after planting, but highly significant increases occurred between 14 and 20 and between 26 and 32 months. More rotenone per acre was produced within each harvest than at previous harvests, usually to a highly significant degree. The highest mean rotenone content existed in roots harvested at 26 months after planting. In table 7 it is shown that no significant differences existed in yields of roots from plants propagated by different types of cuttings. Plants from treated leafy cuttings, however, produced significantly more rotenone per acre than plants from medium leafless cuttings. Roots from control leafy and large leafless cuttings developed a greater mean rotenone content than roots from medium leafless cuttings to a highly significant degree. The roots from control leafy cuttings also had a significantly greater mean rotenone content than roots from small leafless cuttings.

TABLE 6.—*Comparison of yields and rotenone content of Derris elliptica harvested at different ages*

Item	Age at harvest				Least significant difference within each yield		
	14 months	20 months	26 months	32 months	*t* at 5 percent	*t* at 1 percent	Rank [2]
Sum [1] of yields per acre for all treatments:							
Roots.....pounds..	855	3,564	3,553	5,216	347	478	32>>26, 20, 14; 26>>14; 20>>14.
Rotenone.....do....	46.8	191.9	235.2	288.6	32.6	45.0	32>>26, 20, 14; 26>20; 26>>14; 20>>14.
Mean rotenone content percent [3]..	5.47	5.38	6.62	5.53	.60	.83	26>>32, 20, 14.

[1] Totals from tables 4 and 5.
[2] The sign ">" indicates significantly greater than (odds 19:1) and the signs ">>" indicate greater than by high significance (odds 99:1).
[3] Rotenone divided by roots multiplied by 100.

TABLE 7.—*Comparison of yields and rotenone content of Derris elliptica grown from different types of cuttings*

Item	Type of cutting					Least significant difference within each yield		
	A Control leafy	B Treated leafy	C Small leafless	D Medium leafless	E Large leafless	*t* at 5 percent	*t* at 1 percent	Rank [2]
Sum [1] or yields per acre for all harvests:								
Roots.....pounds..	2,574	2,822	2,654	2,574	2,564	311	429	None.
Rotenone.....do....	160.7	163.9	145.4	134.1	158.4	28.9	40.1	B>D.
Mean rotenone content percent [3]..	6.24	5.81	5.48	5.28	6.18	.71	.97	A, E>>D; A>C.

[1] Total from tables 4 and 5.
[2] The sign ">" indicates significantly greater than (odds 19:1) and the signs ">>" indicate greater than by high significance (odds 99:1).
[3] Rotenone divided by roots multiplied by 100.

The roots of each plot from the field harvests were separated while fresh into diameter ranges of less than 4, 4 to 8, and over 8 mm. The average yield per acre of dry roots in each diameter class was computed. The data are presented in table 8. The average proportion of roots

TABLE 8.—*Average yield per acre of dry roots of Derris elliptica in three diameter ranges grown from different types of cuttings and harvested at several ages*

Age at harvest (months)	Diameter of roots	Type of cutting					Average
		Control leafy	Treated leafy	Small leafless	Medium leafless	Large leafless	
	Millimeters	*Pounds*	*Pounds*	*Pounds*	*Pounds*	*Pounds*	*Percent*
8	0–4	7.6	10.1	2.8	3.5	5.6	52.1
	4–8	8.6	9.3	1.0	1.7	6.6	47.9
	8+	0	0	0	0	0	0
14	0–4	70.5	68.8	75.5	100.0	92.6	47.6
	4–8	45.0	60.2	45.3	66.7	88.7	35.8
	8+	34.5	43.0	30.2	18.3	15.7	16.6
20	0–4	192	212	159	195	184	26.6
	4–8	192	301	327	305	234	38.0
	8+	301	301	239	225	197	35.4
26	0–4	162	199	154	188	194	25.2
	4–8	285	258	238	305	248	37.6
	8+	285	282	252	232	271	37.2
32	0–4	322	340	386	329	374	33.6
	4–8	363	395	408	366	416	37.4
	8+	322	362	340	244	249	29.0

in each class was expressed on a percentage basis for the different harvests in the same table and are shown graphically in figure 4. There were no roots over 8 mm. in diameter from plants harvested 8 months after planting. At that time the majority of the roots were less than 4 mm. in diameter. In later harvests differences in amounts of roots in the three diameter classes approached equality. These data are of interest because the roots over 8 mm. in diameter are usually somewhat lower in percentage of rotenone plus rotenoids than smaller roots (*5*). However, generally about one-third of the total yield of roots harvested at 20 months or later were larger than 8 mm. in diameter and their exclusion would have materially reduced the total yields.

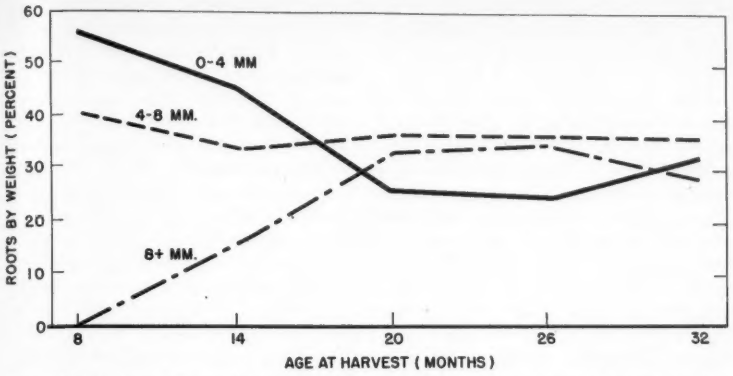

FIGURE 4.—Average percentage of roots within diameter ranges based on dry weight in relation to age at harvest.

DISCUSSION AND CONCLUSIONS

The rotenone and rotenoid content as indicated by the red-color values of *Derris* roots is correlated more closely with insect kill than the percentage of rotenone alone (*4*). This test also requires less time and chemicals than a standard rotenone analysis. In this experiment differences in the content of rotenone plus rotenoids of roots from the various types of cuttings occurred only in roots harvested up to and including 20 months after planting. Within harvests made at 26 and 32 months after planting no significant differences in the content of rotenone plus rotenoids of roots from different types of cuttings occurred. Therefore, the content of rotenone plus rotenoids of roots from the older plants was not affected by the type of cutting used for propagation.

The highest mean rotenone content was attained at the harvest made 26 months after planting. During the 6 months of wet season preceding that harvest no significant increase in weight of roots occurred. This decrease in rate of root growth appears to have been associated with an increase in concentration of rotenone. During the subsequent 6 months of dry season rapid root growth occurred, but the roots harvested 32 months after planting were definitely lower in rotenone than those harvested at 26 months.

The total chloroform extractives include rotenone, rotenoids, and similar substances. Therefore, it is not surprising that averages of these analyses bear about the same relation to each other as rotenone and rotenone plus rotenoids. There were no significant differences among types of cuttings within the harvest made 32 months after planting.

The larger yields of roots from treated leafy cuttings during the first 2 months after planting was a response to the treatment with 3-indolebutyric acid. However, there seems to be no practical value in its use from the standpoint of root yield because field harvests would never be made at this early age and differences did not occur at 14

months and thereafter. The production of more roots from leafy than from leafless cuttings during the first 8 months after planting indicates that the leaves function advantageously in inducing root growth. However, regardless of the type of cutting used, differences in yield did not occur at 14 months and thereafter. On the other hand, there was a lower mortality among small leafy cuttings treated with 3-indolebutyric acid than among control leafy cuttings. The large leafless cuttings had a lower mortality than medium or small leafless cuttings.

Root development during the first 8 months was retarded by the hot dry weather. The rains which occurred between the harvests at 8 and 14 months were associated with considerable increase in root development. Although relatively little rain fell between the harvests at 14 and 20 months, root development was rapid. Probably the roots had grown to such depths by that time that low rainfall was not a limiting factor. During the next 6-month interval there was no significant increase in root weights. This may be explained by the growth flushes which occur in field plantings of this variety of *Derris* (*7*, *9*). Thus, for a period of a few months the tops may make no marked growth. Then there follows a flush of new stems and leaves accompanied by slow root growth. The reverse of this situation apparently occurs when the roots increase rapidly simultaneously with slow top development. For example, during the period between the harvests made at 14 and 20 months relatively little top development was observed, whereas there was a large increase in roots. In the next 6 months considerable top growth occurerd but there was no significant increase in roots. In the last 6 months top development appeared to be slow while roots increased considerably. During periods of slow root development and rapid top growth a marked increase in the relative concentration of rotenone occurred. However, in spite of a decrease in concentration of rotenone between 26 and 32 months the increase in root weight resulted in a much greater yield of rotenone per acre than from previous harvests.

Any increase in weight of roots resulted in an increase in the total yield of rotenone provided the percentage of rotenone did not decrease correspondingly. Therefore, it is not surprising to note the continuous increase in total yield of rotenone up to the harvest made 32 months after planting. From a practical standpoint *Derris* should not be harvested until the total profit per acre per month ceases to increase. This is calculated on an acre basis as follows: The value of the rotenone produced in n months divided by n gives the gross return per month. The total cost of production—planting, weeding, and harvesting—divided by n gives the cost per month. The difference is net return per month. This value increases at least to 32 months and presumably would reach a maximum at some time thereafter and then decrease. The harvest should be made at the time of maximum net return.

The percentage of roots in different diameter groups is of interest, for large roots may contain only traces of rotenone and rotenoids. In the last two harvests of this experiment composite samples of roots produced from all the different types of cuttings contained at least 5 percent of rotenone which would meet buyer specifications. In some

cases, however, it might be necessary to discard at least a portion of the large roots in order to have the required percentage of rotenone in composite samples.

SUMMARY

An experiment was performed to establish the relation between yield of roots, rotenone, rotenone plus rotenoids, and chloroform extractives of *Derris elliptica* propagated from five types of cuttings and harvested at intervals up to 32 months.

The following types of stem cuttings of the Sarawak Creeping variety of *D. elliptica* were used: Small leafy cuttings treated with 3–indolebutyric acid; similar cuttings not treated; and large, medium, and small leafless cuttings not treated. Replicate samples of roots were harvested 24 days, 2, 3, 8, 14, 20, 26, and 32 months after the cuttings were planted. Roots of later harvests were separated into diameter groups of less than 4, 4 to 8, and more than 8 mm. The dried roots were analyzed for rotenone, rotenone plus rotenoids, and total chloroform extractives.

Among types of cuttings within harvests no differences existed in the average content of rotenone plus rotenoids 26 and 32 months after planting. Differences in rotenone content occurred in all harvests except that at 26 months, and differences in total chloroform extractives existed in all harvests except at 32 months.

The largest percentage of rotenone occurred in roots harvested 26 months after planting.

No differences in yields of roots among the types of cuttings within each harvest occurred 14 months after planting and thereafter.

Small and medium leafless cuttings produced roots inferior to those from control leafy or large leafless cuttings.

The total yield of rotenone from each harvest was significantly higher than that from the previous harvest and, therefore, the optimum age at harvest is 32 months or longer after the planting of cuttings.

Approximately one-third of the total weight of the later harvests consisted of roots larger than 8 mm. in diameter, but their inclusion did not reduce the rotenone below 5 percent.

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THE EFFECT OF BENZENE HEXACHLORIDE ON SOIL ORGANISMS [1]

By J. K. Wilson, *professor of soil technology*, and R. S. Choudhri, *Government of India scholar, New York (Cornell) Agricultural Experiment Station*

INTRODUCTION

Many chemical compounds are being recommended for the control of weeds, brush, insects, and eelworms, and the use of such agents is increasing rapidly. Compounds, such as borax, ammonium sulfamate, dinitro selective sprays and several 2,4-D formulations for killing weeds and brush, sodium pentachlorphenate for fungi, chlordan and DDT for insects, D-D and ethylene dibromide for nematodes and wireworms, TDE for larvae of *Anapheles*, and sabadilla for corn earworm have been tried among others. If such compounds are toxic to these various forms of life, it is conceivable that they may also affect adversely the desirable microflora of the soil.

A note concerning the effect of DDT on the population and activities of the flora of the soil has recently been published by the authors.[2] It shows that any reasonable application of DDT to soil has little or no effect on the well-being of such flora. Lewis and Hamner [3] found that 2,4–D applied at normal rates for killing weeds also had no important effect on soil micro-organisms or on plant pathogens present in the soil. Recently Carlyle and Thorpe [4] investigated the effect of ammonium and sodium salts of 2,4–D on *Rhizobium* bacteria and observed no serious inhibiting effect on the growth of any of the rhizobia until a concentration of 0.03 percent, which would correspond to an application of 200 pounds per acre, was reached. Stevenson and Mitchell [5], on the contrary, showed that 0.02 percent 2,4-dichlorophenoxyacetic acid or its sodium salt in potato-dextrose agar had a decidedly retarding effect on four different bacteria, but even concentrations of the order of 0.08 percent had no apparent effect on three different fungi.

It seems possible, therefore, that certain of these chemical agents may be injurious to one or more members of the various physiological

[1] Received for publication September 6, 1947.

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groups of micro-organisms. This paper records the results of studies of the effect of benzene hexachloride—a toxic substance for wireworms—on certain autotrophic and heterotrophic organisms that inhabit the soil and on the growth of plants representing four genera of legumes.

MATERIAL

The material commonly designated as benzene hexachloride[6] is 1,2,3,4,5,6–hexachlorocyclohexane, the chemical formula of which is $C_6H_6Cl_6$. It comprises several isomers and possesses a disagreeable, musty or moldy odor and is irritating to the eyes, nose, and skin. The preparation used in this investigation was a wettable powder containing 2.5 percent of the gamma isomer and a total isomer content of 20 percent of the crude product. This material as well as five of the purified isomers (alpha, beta, gamma, delta, epsilon) and heptachlorocyclohexane were employed in various experiments recorded herein. The last-named substance occurs in all of the commercial crude mixtures and is responsible for most of the odor.

TESTS TO DETERMINE THE EFFECTS OF CRUDE BENZENE HEXACHLORIDE

EFFECT ON THE PRODUCTION OF AMMONIA IN SOIL

A test to determine the effect of benzene hexachloride on the production of ammonia was made by analyzing soil to which the insecticide was added. The desired quantity was mixed with dry soil to which certain compounds of high nitrogen content (casein, peptone, urea, and dried blood) had been thoroughly incorporated. The moisture was adjusted in tumblers so that 100 gm. of treated soil weighed after watering 125 gm. The tumblers were covered and held at room temperature for 7 days. Daily weighings and the addition of distilled water, making up the loss, kept the moisture fairly uniform.

TABLE 1.—*Effect of benzene hexachloride on the production of ammonia in duplicate samples of soil*

Treatment[1]	Milliliters of 0.1597 N acid required to neutralize the ammonia	Treatment[1]	Milliliters of 0.1597 N acid required to neutralize the ammonia
Soil only	0.27	Soil with urea	14.43
Soil with benzene hexachloride	.30	Soil with urea and benzene hexachloride	13.34
Soil with casein	4.72	Soil with dried blood	.80
Soil with casein and benzene hexachloride	5.11	Soil with dried blood and benzene hexachloride	.81
Soil with peptone	5.33		
Soil with peptone and benzene hexachloride			

[1] Additions where used were 0.5 gm. of material to be ammonified and 0.15 gm. of 20 percent crude benzene hexachloride.

[6] Crude benzene hexachloride was supplied by E. I. du Pont de Nemours & Co. (for use in 1946), heptachlorocyclohexane (also a crude product) by the California Spray-Chemical Corp., and the epsilon isomer by Dow Chemical Co. Other purified isomers were supplied by Dr. L. B. Norton, of the Department of Entomology, Cornell University.

At the end of this period the soil was extracted with dilute hydrochloric acid, an aliquot was neutralized with sodium hydroxide, and the ammonia distilled into standard sulfuric acid. By this method it was possible to evaluate the effect of benzene hexachloride on the production of ammonia. A summary of the findings is presented in table 1.

Somewhat similar values with and without the application of benzene hexachloride, using different materials to be ammonified, show that the treatment has no striking effect on the production of ammonia in soil by the ammonifiers.

EFFECT ON THE POPULATION OF ORGANISMS AS DETERMINED BY COUNTING COLONIES ON AGAR IN PETRI DISHES

This test was performed by adding 0.15 percent of the 20 percent crude benzene hexachloride to air-dry Dunkirk fine sandy loam. This amount is approximately 100 times as much per acre as is ordinarily recommended to eradicate wireworms. After thoroughly mixing, the moisture was adjusted so that 100 gm. of the treated soil weighed 125 gm. This moisture was maintained for 7 days, at which time the population of bacteria and fungi was determined. The medium employed in this test is commonly referred to as soil-extract-glucose-agar. A summary of the findings is shown in table 2.

TABLE 2.—*Effect of benzene hexachloride on population of organisms*

[Organisms in millions per gram of soil]

Treatment	Bacteria	Fungi
Control	3.6	1.1
Treated with 0.15 percent of 20 percent benzene hexachloride	3.5	1.0

These counts of bacteria and fungi in soil that had received an addition of benzene hexachloride are self-explanatory and show that a heavy application of the crude insecticide had little or no harmful effect on the population of these organisms.

Plots of field soil growing potatoes which had received a treatment of benzene hexachloride in varying amounts were made available for sampling and examination by the Department of Entomology of the University. The soil is a Wooster type. Samples from these plots representing the surface 6 inches were taken on July 25, 1946, in order to ascertain the effect of benzene hexachloride on actinomyces, bacteria, and fungi. The potatoes were showing fairly well in rows. The samples from plots receiving the heavier applications still contained the characteristic odor. The moisture content of samples from the different plots, representing different applications of benzene hexachloride, ranged from 13 to 16 percent. A summary of the data is presented in table 3.

The counts shown in table 3 reflect the lack of uniformity characteristic of soil under field conditions, but at the same time they indicate the nontoxic action of benzene hexachloride on bacteria and fungi. The heaviest application (32 pounds per acre) of this agent still maintained bacterial counts more or less identical with the controls, and that of the fungi was even slightly higher.

TABLE 3.—*Effect of benzene hexachloride on certain organisms*

[Numbers represent organisms in 1/10000 of a gram of soil]

Treatment	Actinomyces [1]		Bacteria [1]		Fungi [1]	
	1	2	1	2	1	2
Control	68	70	105	90	24	22
4 pounds per acre of benzene hexachloride	53	50	97	87	26	42
8 pounds per acre of benzene hexachloride	53	47	124	90	17	47
16 pounds per acre of benzene hexachloride	56	42	72	140	36	18
32 pounds per acre of benzene hexachloride	51	23	105	107	43	34

[1] 1 and 2 show results of duplicate observations as averages of 4 plates each.

EFFECT ON AZOTOBACTER

The above method used to determine the micro-organic population of the soil did not measure the effect of benzene hexachloride on *Azotobacter*, and therefore it appeared desirable to make additional tests. To this end, the same samples of field soil that were collected to determine the effect of this agent on the organisms referred to above were used. The soil from each of the various plots was mixed with about 2 percent of sugar and about 1 percent of magnesium ammonium phosphate. Enough water was added so that when thoroughly kneaded the soil became a thick paste. This was then smoothed in a suitable dish and incubated about 36 hours. Counts of colonies on the surface of the plaque gave a measure of the effect of the agent on the population of *Azotobacter*-like colonies. The following results were obtained.

Treatment	*Number of Azotobacter-like colonies per square millimeter (average of 10 readings)*
Control	7. 6
4 pounds an acre of benzene hexachloride	5. 1
8 pounds an acre of benzene hexachloride	1. 4
16 pounds an acre of benzene hexachloride	. 9
32 pounds an acre of benzene hexachloride	3. 0

A second determination with these same samples gave almost identical results. The results indicate that an application of as little as 4 pounds an acre of the formulation containing 20 percent of benzene hexachloride reduced the number of *Azotobacter*-like colonies that appeared on plaques, thus causing injury to this nitrogen-fixing organism. Since the samples were taken from field plots where the material was used in actual practice the effect obtained by an application of 32 pounds an acre may be ascribed to sampling error. Otherwise the heavier the application the greater was the injurious effect.

EFFECT ON THE GROWTH AND NODULATION OF LEGUMES

Small crocks were used to hold 1,200 gm. of Dunkirk fine sandy loam. To this soil benzene hexachloride was added. The applications were in amounts of 0, 3, 10, 30, 100, 500, and 1,000 p. p. m. excluding the carrying agent and were thoroughly mixed with the soil. The appropriate bacteria for the legumes to be grown were suspended in the distilled water used to bring the moisture to 20 percent of the

dry weight of the soil, and the moisture was maintained by daily additions of water. Final observations on the growth of the plants and on the nodulated condition of the roots were made on the thirtieth day.

It was apparent that an application of 30 p. p. m. was seriously injurious to red clover (*Trifolium pratense*), soybeans (*Glycine max*), alfalfa (*Medicago sativa*), and hairy vetch (*Vicia villosa*). Even the injurious effect of 3 parts in a million could be noted. The heavier the application the more severe was the injury. Hardly any growth occurred where 500 or 1,000 p. p. m. were present. It was observed, however, that where lateral roots developed nodules were invariably present on the roots. Apparently the bacteria for these species of legumes tolerated a heavier application of the benzene hexachloride than did the green plants. Carlyle and Thorpe[7] arrived at the same conclusion with regard to 2,4-D salts.

The soil in which these plants grew was retained in the moist condition for 30 additional days and seeded again to the same four species of legumes. Insofar as could be ascertained, the results were identical with those of the first test. It was observed also that the characteristic musty odor of the insecticide had not disappeared from the soil even after 90 days.

EFFECT ON THE GROWTH OF PURE CULTURES OF CERTAIN ORGANISMS ON AGAR SLOPES IN TEST TUBES

Two procedures were followed in making tests to determine the effect of benzene hexachloride on the growth of certain pure cultures in test tubes. In one case the crude material was broadcast on the surface of the slopes with a sterile loop. On top of this the organism was smeared. In the other case the organism was smeared on the surface of the slope and then the insecticide was broadcast over the smear. Controls were maintained in all cases. In some instances contaminations from the unsterilized benzene hexachloride occurred. These appeared to be caused mostly by *Aspergillus niger*. The bacteria from the nodules of alfalfa, red clover, and hairy vetch, four strains of fungi (*Aspergillus niger, A. oryzae, Penicillium expansum, P. italicum*), and three species of algae including *Chlorella vulgaris* were used. Observations concerning the character of growth were made often over a period of 4 weeks or longer.

Growth of the bacteria and of the fungi seemed to be somewhat retarded during the first 24 hours, but on subsequent examination it appeared to be equal to or even somewhat better in certain cases than that of the controls. Sometimes the growth seemed to be patchy, but within 2 or 3 days it covered the entire spreading of the benzene hexachloride. In cases where the growth was either slow or patchy it appeared as though the insecticide functioned as a mechanical barrier rather than as a toxic agent.

The algal cultures were transferred to a suitable medium and illuminated with light from an electric bulb. The growth and multiplication of these algal cultures were definitely suppressed by the presence of benzene hexachloride; no growth was observed even after a lapse of several weeks. Growth of the check cultures, however, was profuse.

[7] See footnote 4, p. 25.

REMARKS

It is apparent from the data that an application of crude benzene hexachloride considerably in excess of the amount recommended to control such organisms as wireworms has no pronounced deleterious effect on certain heterotrophic micro-organisms of the soil. It may be toxic, however, to those organisms that effect the oxidation of sulfur or to those that bring about certain other physiological processes, such as nitrogen fixation, and it does appear to be exceedingly toxic to members of four genera of legumes (red clover, soybean, alfalfa, and hairy vetch) and to various species of algae. From these latter findings it was suspected that the crude benzene hexachloride contained copper or traces of other metals that were injurious, but tests for copper, lead, and zinc gave no indication of their presence. Since there can be several isomers of benzene hexachloride, it is possible that a certain one or a combination of several in the crude insecticide employed might account for the toxicity to certain micro-organisms and higher plants.

TESTS TO DETERMINE THE EFFECT OF PURE ISOMERS OF BENZENE HEXACHLORIDE AND HEPTACHLOROCYCLOHEXANE ON THE GROWTH OF CERTAIN ORGANISMS

Each isomer, alpha, beta, gamma, delta, and epsilon, was broadcast in sufficient quantity on the surface of agar slopes so that the surface of each was white with crystals and each slope was then smeared with an organism. In comparable tubes the organisms were first spread on the surface of the slopes and the isomers broadcast over them. In both cases the applications were heavy. Several strains of bacteria, fungi, actinomyces, and algae were employed. The effect of these isomers on the organisms is given in table 4.

There was no indication of toxicity at the end of 14 days in any instance where the isomers alpha, beta, or epsilon were used. In certain cases increased growth appeared where these isomers were present. The gamma isomer also proved to be nontoxic in most cases, but invariably caused subnormal growth of the three species of algae.

TABLE 4.—*Effect of pure isomers of benzene hexachloride and of heptachlorocyclohexane on the growth of certain organisms* [1]

Organism	Control	Alpha	Beta	Gamma	Delta	Epsilon	heptachlorocyclohexane
Rhizobium leguminosarum	+	+	+		+	+	+
Rhizobium japonicum	+	+	+	+	±		±
*Rhizobium sagittalis**	+	+	+	+	±	++	+
Azotobacter chroococcum	+	+	+	+	+		+
Bacillus mycoides	+	+	+	+	+	+	
Denitrifying bacteria	+				−+		−+
Urea-decomposing bacteria	+				−		−
Aspergillus niger	+				+		+
Actinomyces scabies	+	+	+	+			
Actinomyces nocardia (saprophytica)	+	+	+	+	±	++	±
Rhizoctonia solani	+				−		−
Chlorella vulgaris	+	++	++	±	−	+	±
Algae—a	+	++	++	±	−	+	−
Algae—b	+			±	±		+

[1] + Indicates normal growth; ++, increased growth; −+, delayed growth; ±, subnormal growth; and − no growth.

*This organism was isolated from *crotalario sagittalis*.

The delta isomer of benzene hexachloride permitted normal growth of *Rhizobium leguminosarum* and of *Azotobacter chroococcum* but delayed the growth of four other organisms. This was apparent after an incubation period of 2 weeks. Fruiting of *Aspergillus niger* was delayed in comparison with that of the controls. The urea-decomposing organisms, *Rhizoctonia solani*, and the three species of algae employed were injured by its presence. There was but little evidence that growth of these organisms had occurred in the presence of this isomer even after several weeks.

Heptachlorocyclohexane appeared to be highly toxic to some organisms and one of three species of algae failed to grow in its presence. (Table 4.)

DISCUSSION

The first tests were planned to determine the effect of benzene hexachloride on the total population and activities of micro-organisms and on the growth performance of certain legumes. The data support the statement that there was little or no harmful effect on the total population or on ammonification in the soil, but applications representing those recommended for the control of wireworms were injurious to certain legumes growing on the soil. Marked tolerance of the legume bacteria to this agent was evident in the formation of nodules on the roots of legumes even under applications heavy enough to interfere with the normal growth of roots.

Other tests were more specifically designed to ascertain the response of certain pure cultures of organisms when in contact with 20 percent crude benzene hexachloride. This agent, although applied much in excess of the amount ever recommended, was not significantly toxic to several species of fungi and bacteria. The toxic effect on *Azotobacter*-like organisms appearing on plaques of soil and the complete suppression of algal growth are some of the instances indicative of the toxic action of benzene hexachloride.

Subsequent experiments were designed to critically evaluate the effect of certain isomers of this compound and of heptachlorocyclohexane, primarily, on the pure cultures referred to above. It was observed that the isomers alpha, beta, and epsilon were practically harmless. It appears, therefore, that much of the toxic action of benzene hexachloride that contained a total of 20 percent of the isomers should be attributed either to the gamma isomer, for it inhibited the growth of algae, or to the delta isomer in the presence of which the growth of algae and several other organisms was scant or even prevented. Heptachlorocyclohexane, which proved to be equally injurious and which is present in almost all crude mixtures of benzene hexachloride, may also be responsible for some of the toxic effects.

Experiments in which pure cultures were employed have shown that the delta isomer, and in some cases the heptachlorocyclohexane, suppressed the growth of several other organisms. These included the urea-decomposing bacteria and *Actinomyces nocardia*. Averages of counts of actinomyces obtained from the soil that received increasing applications of the crude benzene hexachloride gave also some indication that the heavier the application the greater was the toxic effect. In the pure culture tests where the crystalline materials were applied in sufficient quantity to make the surface of the slopes white, the

differences apparent may be attributed to properties of the individual isomers.

Such data may mean that certain groups of organisms may be slightly suppressed by one or more of the isomers or by the impurities and that the effect is not severe enough to be clearly reflected in a count of total organisms.

If benzene hexachloride is as toxic to *Nostoc muscorum*, a nitrogen-fixing alga, as it has proved to be to *Chlorella vulgaris* and to *Azotobacter chroococcum*, a partial or complete loss of such organisms from the soil cannot easily be ignored. The injurious effect of this agent on certain legumes should be emphasized also.

The crude benzene hexachloride employed in this study appeared highly toxic to *Azotobacter*, but not any of the purified isomers interfered with normal growth of *A. chroococcum* on agar slopes even though heavy applications were made. Possibly other species of this genus may be more susceptible to applications of benzene hexachloride, to one or more of the purified isomers, or to some unidentified impurity.

SUMMARY AND CONCLUSION

The effect of benzene hexachloride on the micro-organic flora of the soil and on a representative of four genera of legumes was ascertained by comparing the presence, growth, and certain physiological processes of these organisms and the growth of plants under conditions where the benzene hexachloride was applied and where it was omitted. The production of ammonia and the total population of actinomyces, bacteria, and fungi were affected very little if any by applications considerably in excess of those commonly employed for the control of wireworms. Chlorella and two other algae failed to survive even where small applications were made. An application of the equivalent of 30 p.p.m. of the toxic material in the crude benzene hexachloride was injurious to the legumes. No such toxic effects were noted when tests were made with the pure alpha, beta, and epsilon isomers. Effect of these isomers was, however, not determined on legumes.

The gamma isomer was slightly toxic to algae. The delta isomer was highly toxic to algae and also to several micro-organisms, including *Rhizoctonia solani*, urea-decomposing bacteria, and *Actinomyces nocardia*. Heptachlorocyclohexane was toxic also to several organisms.

None of the purified isomers or heptachlorocyclohexane was harmful to *Azotobacter*. The crude form of benzene hexachloride however, was toxic, to this organism. The injurious effect of the crude benzene hexachloride is due probably to the gamma and delta isomers, to heptachlorocyclohexane, to some unidentified or uninvestigated isomer, to a combination of two or more isomers, or to some impurity.

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